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Remarks:
The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(54) A regulatory gene for the expression of nitrile hydratase gene

(57) The invention relates to a regulatory gene coding for a polypeptide having the ability to activate a promoter for a nitrile hydratase gene, a recombinant DNA containing said regulatory gene, and a transformant transformed with said recombinant DNA.

The introduction of the regulatory gene of the invention along with a nitrile hydratase gene and its promoter region permits bacteria of the genus Rhodococcus to produce a higher level of nitrile hydratase. Other extraneous gene can also be introduced into a region downstream of the promoter to produce other proteins in high yield.

EP 0 713 914 A2

Description

FIELD OF THE INVENTION

The present invention relates to a regulatory gene derived from bacteria of the genus Rhodococcus and coding for a polypeptide capable of activating a promoter for a nitrile hydratase gene, a recombinant DNA containing said DNA, and a transformant transformed with said recombinant DNA.

BACKGROUND OF THE INVENTION

Processes for producing amides by nitrile hydratase that is an enzyme hydrating nitriles to amides came to be used as industrially advantageous methods by virtue of the reaction at normal temperature and pressure, higher conversion degree, etc. In particular, bacteria of the genus Rhodococcus are known to accumulate a significant amount of the enzyme in their cells to exhibit high catalytic activity (see Japanese Patent Publication No. 4873/1992 and Japanese Laid-Open Patent Publication Nos. 91189/1987; 470/1990 and 84198/1990).

As compared with such conventional processes, those using a nitrile hydratase gene cloned by genetic recombination are expected to attain drastic improvements in the catalytic ability of bacteria to hydrate nitriles because the bacteria can be engineered to contain multiple copies of the same gene. Further improvements in the enzyme are also facilitated by genetic recombination techniques such as site-specific mutagenesis, random mutagenesis, etc. A nitrile hydratase gene derived from the genus Rhodococcus has been obtained from N-774 (Japanese Laid-Open Patent Publication No. 119778/1990) and J-1 (Japanese Laid-Open Patent Publication No. 211379/1992). The nitrile hydratase gene from N-774 could successfully inserted into a Rhodococcus-E. coli hybrid plasmid vector (Japanese Laid-Open Patent Publication No. 64589/1993) and attained high activity when introduced into bacteria of the genus Rhodococcus (Japanese Laid-Open Patent Publication No. 68566/1993). On the other hand, there is no report on the expression of the nitrile hydratase gene derived from Rhodococcus rhodochrous J-1 in bacteria of the genus Rhodococcus, in spite of its higher nitrile hydratase activity than N-774.

SUMMARY OF THE INVENTION

The present inventors speculated that the failure to express the gene derived from J-1 results from the absence in the DNA fragment of a regulatory gene involved in function of a promoter for the nitrile hydratase gene. Hence, they sought for the regulatory gene in total DNA from J-1 and found the gene located upstream of the nitrile hydratase structural gene. This gene was successfully used for the high-level expression of nitrile hydratase in a transformant of the genus Rhodococcus.

That is, the present invention relates to a regulatory gene derived from bacteria of the genus Rhodococcus and coding for a polypeptide capable of activating a promoter for the nitrile hydratase gene, a recombinant DNA containing said DNA, and a transformant transformed with said recombinant DNA.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the construction scheme of the recombinant plasmid pHJK18.
Fig. 2 shows the construction scheme of the recombinant plasmid pHJK19.

DETAILED DESCRIPTION OF THE INVENTION

Hereinafter, the present invention is described in detail. The present invention is practiced in the following steps.

(1) Preparation of total DNA:

Total DNA is isolated from Rhodococcus rhodochrous J-1.

(2) Construction of a DNA Library:

The fragment of the target nitrile hydratase gene is excised from a plasmid containing the nitrile hydratase gene from J-1 and then labeled with a radioisotope. This fragment is used as a probe.

The total DNA obtained in step (1) is cleaved with restriction enzymes and subjected to Southern hybridization (Southern E. M., J. Mol. Biol. 98, 503 (1975)) with the probe prepared above. The detected DNA fragments containing the target gene are inserted into a plasmid vector pUCI9 to prepare a library.

(3) Preparation of transformants and selection of a recombinant DNA:

The recombinant DNA library constructed in step (2) is used for preparation of transformants from which a colony carrying the target recombinant DNA is selected by colony hybridization (R. Bruce Wallace et al., Nuc. Acids Res. 9, 879 (1981)) using the probe obtained in step (2). The hybridized colony is further subjected to Southern hybridization so that the presence of the target recombinant DNA is confirmed.

(4) Isolation and purification of a recombinant plasmid and construction of a restriction enzyme map:

A recombinant plasmid is isolated from the transformant obtained in step (3). This plasmid is designated plasmid pNHU10. It is cleaved with various restriction enzymes and analyzed by electrophoresis to prepare a restriction enzyme map.

(5) Construction of a recombinant plasmid by inserting a fragment containing a regulatory gene and the nitrile hydratase gene into a plasmid vector capable of replicating in the genus Rhodococcus:

Recombinant plasmids pHK18 and pHK19, each carrying a regulatory gene and a fragment containing a nitrile hydratase gene, are constructed by inserting the plasmid obtained in step (4) and plasmid pNHJ10H containing the nitrile hydratase gene derived from J-1 into the hybrid plasmid vector pK4.

(6) Transformation of bacteria of the genus Rhodococcus and production of nitrile hydratase by the transformant:

The plasmid obtained in step (5) is introduced into bacteria of the genus Rhodococcus, and the expression of the nitrile hydratase in the transformant is confirmed.

(7) Deletion-plasmids and nitrile hydratase activity:

Deletion-plasmids are prepared by deletion of various regions from the plasmid obtained in step (5). The resultant deletion-plasmids are used to identify which region is essential for the expression of nitrile hydratase. The region essential for the expression of nitrile hydratase, but not encoding nitrile hydratase, is thus identified.

(8) Nucleotide sequencing:

The nucleotide sequence of the region identified in step (7) is determined.

Rhodococcus rhodochrous J-1 has been deposited as FERM BP-1478 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan. Transformant TG1/pNHJ10H (FERM BP-2777) containing plasmid pNHJ10H, transformant Rhodococcus rhodochrous ATCC12674/pK4 (FERM BP-3731) containing the hybrid plasmid vector pK4, and transformant JM109/pNHU10 (FERM BP-5224) containing plasmid pNHU10 have been deposited as substitutes for plasmid pNHJ10H, hybrid plasmid vector pK4, and plasmid pNHU10, respectively.

EFFECT OF THE INVENTION

The introduction of the regulatory gene of the invention along with the nitrile hydratase gene and its promoter region permits bacteria of the genus Rhodococcus to produce a higher level of nitrile hydratase. Other extraneous gene can also be introduced into a region downstream of the promoter to produce other proteins in high yield.

PREFERRED EMBODIMENTS OF THE INVENTION

Hereinafter, the present invention will be illustrated in detail by reference to the following Example, which however is not intended to limit the scope of the invention.

The following abbreviations are used in Example.

TE: 10 mM Tris-HCl (pH 7.8)-1 mM EDTA (pH 8.0)
TNE: 50 mM Tris-HCl (pH 8.0)-1 mM EDTA (pH 8.0)-50 mM NaCl
STE: 50 mM Tris-HCl (pH 8.0)-1 mM EDTA (pH 8.0)-35 mM sucrose
2×YT medium: 1.6 % trypton, 1 % yeast extract, 0.5 % NaCl
MY medium: 1 % polypeptone, 0.3 % yeast extract, 0.3 % malt extract, 1 % glucose

(1) Preparation of total DNA

Rhodococcus rhodochrous J-1 (FERM BP-1478) was incubated at 28 °C for 2 days in 100 ml medium (glucose, KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, yeast extract, peptone, $CoCl_2$, urea, 1 L water, pH 7.2). The bacterial cells were harvested by centrifugation and the pellet was washed with TNE and suspended in 10 ml TE. 4 ml of 0.25 M EDTA, 10-20 mg lysozyme, 10-20 mg achromoprotease and 10 ml of 10 % SDS were added to the suspension. The suspension was allowed to stand at 37 °C for 3 hours and then centrifuged. 0.7 ml of 2.5 M sodium acetate and diethyl ether were added to 20 ml of the supernatant. The upper layer was discarded, and 2 volumes of ethanol was added to the lower layer. The DNA precipitates were recovered with a grass rod. The DNA was rinsed for 5 minutes each with TE:ethanol 2:1, 1:9 and 0:10 (v/v) and then dissolved in 2-4 ml TE. 10 µl of a mixture of RNase A and T1 was added to the solution and the mixture was incubated at 37 °C. An equal amount of TE-saturated phenol was added to the mixture which was then centrifuged. More than equal amount of ether was added to the upper layer, and the lower layer was saved. The lower layer was dialyzed overnight against 2 L TE containing a small amount of chloroform and further dialyzed for 3-4 hours against fresh TE buffer. 4 ml of crude total DNA was obtained.

(2) Construction of a DNA library

10 µl of plasmid pNHJ10H in which a 6.0 kb DNA fragment containing the H-type nitrile hydratase gene from J-1 had been inserted into vector pUC19 (Japanese Laid-Open Patent Publication No. 211379/1992; Biochim. Biophys. Acta 1129, 23-33 (1991)) was cleaved at 37 °C for 2 hours with a mixture of 2 µl each of restriction enzymes Sac I and Eco RI, 3 µl of reaction buffer (10×) and 13 µl sterilized water, and the digest was electrophoresed on 1 % agarose gel. The Sac I-Eco RI fragment, 0.37 kb long, was cut off from the gel and labeled with radioisotope ^{32}P.

The total DNA from J-1 obtained in step (1) was digested with Eco RI and Eco RV, electrophoresed on an agarose gel and subjected to Southern hybridization where the 0.37 kb Sac I-Eco RI fragment prepared above was used as the probe. An about 4.3 kb DNA fragment containing a region upstream from the nitrile hydratase gene was hybridized with the probe. A fraction containing the 4.3 kb DNA fragment was eluted from the agarose gel.

Separately, 10 µl plasmid vector pUC19 was digested at 30 °C for 2 hours with a mixture of 2 µl each of restriction enzymes Sma I and Eco RI, 3 µl reaction buffer (10×), and 13 µl sterilized water. The Sma I- and Eco RI-cleaved pUC19 was purified as follows: After an equal amount of TE-saturated phenol was added to the reaction solution, the solution was stirred and separated into upper (aqueous) and lower layers by centrifugation. The upper layer was extracted again with TE-saturated phenol in the same manner and further extracted twice with an equal amount of chloroform in the same manner. 3 µl of 3 M sodium acetate and 90 µl ethanol were added to the upper layer, and the sample was allowed to stand at -80 °C for 30 minutes, centrifuged, dried and dissolved in TE.

A DNA library was prepared by inserting 3 µl of the above fraction containing the 4.3 kb fragment from the total DNA into the above Sma I- and Eco RI-cleaved pUC19 by the action of TAKARA ligation kit overnight at 4 °C.

(3) Preparation of transformants and selection of a recombinant DNA

E. coli JM109 (available from Takara Shuzo Co., Ltd.) was inoculated into 10 ml of 2×YT medium and incubated at 37 °C for 12 hours. After incubation, the resultant culture inoculated into fresh 2×YT medium to a concentration of 1 %, and the mixture was incubated for 2 hours. The culture was centrifuged and the pellet was suspended in 5 ml of cold 50 mM $CaCl_2$. The suspension was placed on ice for 40 minutes and then centrifuged again, the pellet was suspended in 0.25 ml of cold 50 mM $CaCl_2$. 60 µl of the recombinant plasmids (DNA library) prepared in step (2) was added to the suspension. The mixture was allowed to stand at 0 °C for 40 minutes and heat-shocked at 42 °C for 2 minutes, followed by addition of 1 ml of 2×YT medium. The mixture was incubated at 37 °C for 60 minutes with shaking. The culture, 100 µl per plate, was spreaded on a 2×YT agar plate containing 50 µg/ml ampicillin. The plate was incubated at 37 °C. The colonies grown on the plate were selected for those carrying a DNA fragment upstream of the nitrile hydratase gene by colony hybridization in the following manner. The colonies grown on the plate were transferred to a nitrocellulose filter and the bacteria were lysed. The DNA was fixed on the filter and hybridized with the probe (0.37 kb fragment) obtained in step (2). The filter was autoradiographed and a colony containing the target recombinant DNA was selected. Additionally, the recombinant plasmid was extracted from the colony, and the Southern hybridization of the recombinant plasmid with the above probe indicated that the selected colony was a transformant carrying the target gene.

(4) Isolation and purification of the recombinant plasmid and construction of a restriction enzyme map

The transformant selected in step (3) was incubated at 37 °C overnight in 100 ml of 2×YT medium, then collected and washed with TNE. 8 ml of STE and 10 mg lysozyme were added to the cells. The mixture was allowed to stand

at 0 °C for 5 minutes. 4 ml of 0.25 M EDTA, 2 ml of 10 % SDS (at room temperature) and 5 ml of 5 M NaCl were added to the mixture. The mixture was allowed to stand at 0 to 4 °C for 3 hours and ultracentrifuged. 1/2 volume of 30 % PEG 6000 was added to the supernatant. The mixture was allowed to stand at 0 to 4 °C overnight and centrifuged again. TNE was added to the pellet to bring the volume up to 7.5 ml. CsCl was added to the suspension and centrifuged to remove proteins. Then, 300-500 mg/ml ethidium bromide was added to the supernatant and the mixture was transferred to a centrifuge tube. The tube was heat-sealed and ultracentrifuged. cccDNA was recovered. More than equal amount of isopropyl alcohol saturated with water was added to cccDNA to remove the ethidium bromide. The DNA sample was dialyzed against TE, resulting in about 3 ml of the purified recombinant plasmid. The recombinant plasmid thus obtained was designated pNHU10. This recombinant plasmid was digested with several restriction enzymes, and the restriction enzyme map thus prepared is shown in Fig. 1.

(5) Construction of a recombinant plasmid by inserting a fragment containing a regulatory gene and the nitrile hydratase gene into a plasmid vector capable of replicating in the genus Rhodococcus

Plasmid pNHU10 obtained in step (4) overlapped with 0.37 kb nucleotides, and further contained 3.9 kb nucleotides upstream from, the nitrile hydratase gene derived from pNHJ10H. A 1.4 kb Fsp I-Eco RI fragment and a 0.9 kb Sca I-Eco RI fragment were excised from pNHU10 and inserted into the hybrid plasmid vector pK4 for E. coli-Rhodococcus bacteria to give plasmids pHJK16 and pHJK17, respectively. Plasmids pHJK18 and pHJK19 were constructed by inserting a 5.9 kb Eco RI fragment derived from pNHJ10H into plasmids pHJK16 and pHJK17, respectively. These steps are illustrated in Figs. 1 and 2, respectively. In pHJK18 of Fig. 1, the thick arrow indicates the location and direction of the regulatory gene found in the present invention, and the thin arrow indicates the location and direction of the genes coding for 2 subunits of nitrile hydratase. In pHJK19 of Fig. 2, the thick arrow indicates the location and direction of the regulatory gene found in the present invention, and the thin arrow indicates the location and direction of the genes coding for 2 subunits of nitrile hydratase.

(6) Transformation of bacteria of the genus Rhodococcus and production of nitrile hydratase by the transformant

Rhodococcus rhodochrous ATCC12674 at the logarithmic growth phase was harvested by centrifugation, washed 3 times with ice-cold sterilized water and suspended in 15 % PEG 6000 (polyethylene glycol 6000) to a final concentration of at least 10^9 cells/ml. 1 µg of plasmid pHK18 DNA or pHK19 DNA was mixed with 100 µl of the bacterial suspension and the mixture was cooled on ice. This mixture of DNA and bacteria was introduced into a gene pulser chamber, cooled on ice and pulsed with a electrostatic capacity of 25 µF, resistance of 400 Ω and voltage of 20 kV/cm.

The bacterial suspension thus treated was placed on ice for 10 minutes and heated at 37 °C for 5 minutes. 1 ml of MY medium was added to the suspension, which was then shaken at 28 °C for 3 hours. The bacterial suspension was spreaded on an MY agar plate containing 50 µg/ml kanamycin and incubated at 25 °C for 2 days. The colony grown on the plate was streaked on another MY agar plate containing kanamycin, and their resistance to kanamycin was ascertained by their growth on the plate.

The resultant Rhodococcus rhodochrous transformants (Rhodococcus rhodochrous ATCC12674/pHJK18 and Rhodococcus rhodochrous ATCC12674/pHJK19) were incubated at 28 °C for 2 days in a medium (10 g glycerol, 5 g peptone, 3 g yeast extract, 3 g malt extract, 1 g KH_2PO_4, 1 g K_2HPO_4, 0.01 g $CoCl_2 \cdot 6H_2O$, 0.75 g or 3.75 g urea (pH 7.0)/1 L medium). The bacterial cells were harvested by centrifugation, and the pellet was washed with 150 mM NaCl and suspended in 0.1 M HEPES-KOH buffer (pH 7.2) containing 0.35 % n-butyric acid. The cells were disrupted by sonication and centrifuged to remove the cell membrane. The cell extract thus obtained was examined for nitrile hydratase activity. The enzyme assay was carried out in a reaction mixture containing 0.5 ml of 100 mM potassium phosphate buffer (pH 7.0), 1 ml of 200 mM acrylonitrile and 0.5 ml of the cell extract diluted with a suitable amount of water. The reaction was carried out at 20 °C for 10 minutes and stopped by the addition of 0.2 ml of 1 N HCl. The amount of acrylamide formed in the reaction mixture was determined by HPLC.

Table 1 shows the nitrile hydratase activities of the cell-free extracts from Rhodococcus rhodochrous ATCC12674/pHJK18 and Rhodococcus rhodochrous ATCC12674/pHJK19. In the table, urea is an inducer for nitrile hydratase.

Table 1

transformant	nitrile hydratase activity		
	urea (g/L)		
	0	0.75	3.75

Continuation of the Table on the next page

Table 1 (continued)

transformant	nitrile hydratase activity		
Rhodococcus rhodochrous ATCC12674/pHJK18	171	279	115
Rhodococcus rhodochrous ATCC12674/pHJK19	180	242	104
	(unit/mg protein)		

(7) Deletion plasmids and nitrile hydratase activity

Because it was estimated that region not involved in the expression of nitrile hydratase still remained in pHJK18 and pHJK19, deletion plasmids were prepared therefrom and examined for nitrile hydratase activity. The result indicated that the regulatory gene involved in the expression of the activity was located within an about 1.9 kb region between the Sca I and Acc III sites.

(8) Nucleotide sequencing

The nucleotide sequence of the region identified in step (7) was sequenced by the chain termination method (Sanger F. Science, 214, 1205-1210 (1980)) using DNA polymerase. The sequence analysis revealed the presence of a long open reading frame coding for the amino acid sequence shown in the Sequence Listing by SEQ ID: No. 1. The nucleotide sequence of the open reading frame is shown in the Sequence Listing by SEQ ID: No. 2.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Sakaya SHIMIZU
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(C) CITY: Kyoto
(E) COUNTRY: Japan
(F) POSTAL CODE (ZIP): 604

(A) NAME: NITTO CHEMICAL INDUSTRY CO. LTD.
(B) STREET: 5-1, Marunouchi 1-chome, Chiyoda-ku
(C) CITY: Tokyo
(E) COUNTRY: Japan
(F) POSTAL CODE (ZIP): 100

(ii) TITLE OF INVENTION: A regulatory gene for the expression of nitrile hydratase gene

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: EP 95306960.6

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 361 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rhodococcus rhodochrous
(B) STRAIN: J-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Met Pro Leu Arg Arg Leu Asn Leu Gly Leu Val Leu Pro Gln Ser Gly
1               5                  10                  15

Pro Ser Gly Ile Phe Gly Pro Ser Cys Gln Ala Ser Ala Glu Tyr Ala
            20                  25                  30
```

Ile Asp Glu Leu Asn Ala Gly Gly Gly Ile Leu Gly Arg Glu Val Thr
35 40 45

Ala Val Phe Val Asp Gly Gly Ala Asp Pro Ser Ala Val Ala Ala Cys
50 55 60

Ile Ala Asp Gln Thr Lys Arg Arg Glu Leu Asp Ala Val Val Gly Trp
65 70 75 80

His Thr Ser Ala Val Arg Arg Arg Ile Val Ser Ala Ile Gly Gly Arg
85 90 95

Ile Pro Tyr Val Tyr Thr Ala Val Tyr Glu Gly Gly Glu Asn Ser Asp
100 105 110

Gly Val Phe Met Thr Gly Glu Val Pro Thr Asn Gln Ile Leu Pro Ala
115 120 125

Leu Glu Trp Met Thr Glu Ile Gly Val Arg Lys Trp Tyr Val Ile Gly
130 135 140

Ser Asp Tyr Val Trp Pro Arg Lys Thr Val Ser Val Ile Arg Glu Phe
145 150 155 160

Leu Ala Ser Asn Gln Leu Pro Ser Arg Gly Arg Ser Asp Val Arg Leu
165 170 175

Ala Ser Cys Glu Phe Leu Ser Leu Gly Thr Ser Asp Phe Thr Ser Thr
180 185 190

Leu Glu Ala Ile Glu Met Ser Gly Ala Asp Gly Val Leu Val Leu Leu
195 200 205

Leu Gly Gln Asp Ala Val Gln Phe Asn Arg Ser Phe Ser Arg Lys Gly
210 215 220

Leu His Arg Asp Ile Val Arg Leu Ser Pro Leu Met Asp Glu Asn Met
225 230 235 240

Leu Leu Ala Ser Gly Ala His Ala Ala His Gly Leu Tyr Ser Val Ser
245 250 255

Gly Phe Phe Glu Cys Leu Val Thr Gly His Ser Met Asp Phe Glu Ser
260 265 270

Arg Tyr Ile Lys His Phe Gly Pro Thr Ala Pro Pro Ile Thr Ser Pro
275 280 285

Gly Glu Ser Cys Tyr Glu Gly Ile Arg Leu Leu Ala Thr Leu Ala Asp
290 295 300

Arg Ala Gly Asp Leu Asp Pro Met Ser Leu Ser Tyr His Ala Asp Arg
305 310 315 320

Thr Leu Asp Tyr Asp Ser Pro Arg Gly His Val Arg Phe Asp Gly Arg
325 330 335

His Leu Ala Gln Asp Met Tyr Ile Ala Arg Ala Asp Gly Val Glu Phe
340 345 350

Asp Val Leu Ala Gln Val Ser His Val
355 360

(3) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1086 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rhodococcus rhodochrous
(B) STRAIN: J-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GTGCCCCTCC GCCGACTGAA CCTGGGCTTG GTGCTACCTC AGAGTGGACC GTCCGGCATT 60

TTCGGTCCGT CATGCCAGGC GAGCGCCGAG TACGCCATCG ATGAGCTCAA CGCGGGCGGC 120

GGAATCCTGG GCCGAGAGGT TACGGCGGTC TTCGTTGACG GGGGCGCGGA CCCGTCCGCC 180

GTAGCAGCAT GCATCGCCGA CCAGACGAAA CGTCGGGAAT TGGACGCCGT AGTCGGGTGG 240

CACACGTCTG CTGTTCGTCG ACGCATCGTG AGCGCCATCG GCGGACGTAT TCCGTATGTC 300

TACACCGCAG TCTACGAGGG CGGCGAGAAC TCCGACGGCG TGTTCATGAC GGGAGAGGTA 360

CCGACGAATC AGATTCTTCC TGCCCTGGAA TGGATGACTG AGATCGGCGT GCGTAAGTGG 420

TATGTCATTG GCAGTGACTA CGTTTGGCCT CGAAAGACTG TCTCGGTCAT TCGCGAATTC 480

CTGGCGTCGA ACCAGCTACC GAGTCGAGGC CGCAGCGACG TTCGACTGGC GTCGTGCGAG 540

TTCTTGTCAC TAGGCACATC CGACTTCACT TCAACGCTCG AAGCAATTGA GATGTCGGGG 600

GCCGATGGCG TTCTCGTCCT CCTCCTCGGC CAGGACGCAG TACAGTTCAA CCGGTCTTTT 660

TCACGGAAAG GGCTGCACCG CGACATCGTC AGACTCAGTC CGCTGATGGA CGAGAACATG 720

CTGTTGGCAA GCGGCGCACA CGCCGCGCAC GGACTCTACT CGGTGTCGGG GTTCTTCGAG 780

TGCCTGGTCA CCGGGCACAG CATGGATTTC GAATCCAGGT ACATCAAGCA CTTCGGTCCG 840

ACCGCCCCGC CGATCACTTC GCCTGGAGAG TCGTGCTACG AGGGCATTCG GCTGTTGGCC 900

ACTCTTGCAG ACCGGGCCGG CGATCTCGAC CCGATGTCTC TGAGCTATCA CGCAGACCGT 960

ACCCTCGACT ACGACAGCCC TCGAGGCCAT GTCCGCTTCG ATGGTCGCCA TCTCGCTCAG 1020

GACATGTACA TCGCGCGGGC TGACGGAGTA GAGTTCGACG TCTTAGCGCA GGTTTCCCAT 1080

GTGTGA 1086

Claims

1. An isolated regulatory gene coding for a polypeptide having the ability to activate a promoter for a nitrile hydratase gene, said polypeptide having the amino acid sequence set out in Seq. ID No. 1.

2. A regulatory gene according to claim 1 which has the nucleic acid sequence set out in Seq. ID. No. 2.

3. A vector comprising a regulatory gene according to claim 1 or 2 operably linked to a promoter.

4. A vector according to claim 3 further comprising a nitrile hydratase gene containing a promoter region.

5. A transformant prepared by the transformation of a cell with a vector according to claim 3 or 4.

6. A transformant according to claim 1 which is a bacteria of the genus Rhodococcus.

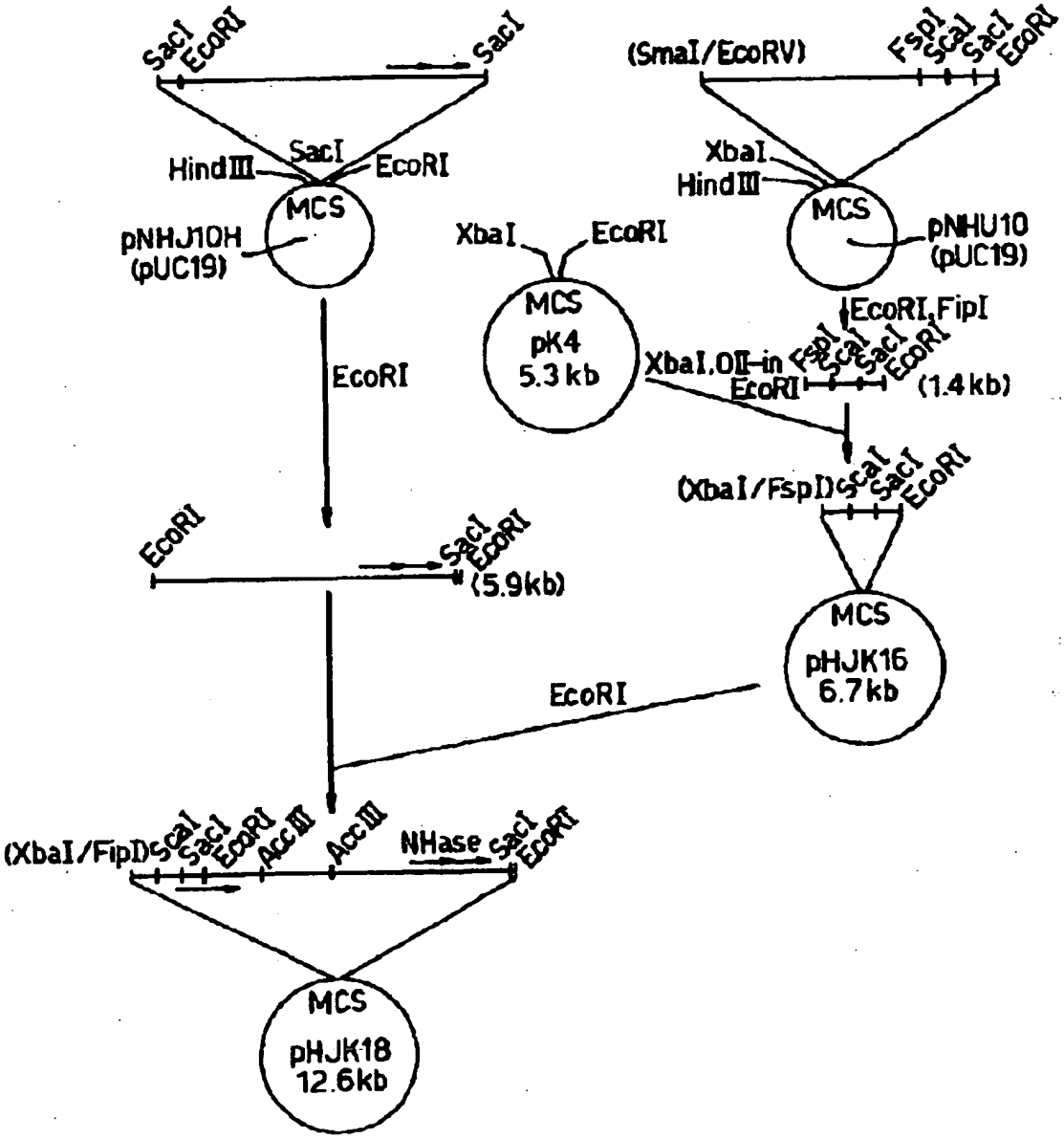
7. A method of preparing an amide by the hydration of a corresponding nitrile which method comprises:

   (a) culturing a transformant according to claim 5 or 6 under conditions in which nitrile hydratase is produced;
   (b) bringing the nitrile into contact with the nitrile hydratase obtained from said culturing under conditions to bring about hydration to an amide; and
   (c) recovering the amide.

8. A method according to claim 7 wherein the nitrile is acrylonitrile.

9. A method according to claim 7 or 8 wherein urea is used as an inducer for nitrile hydratase.

Fig.1.


SacI
EcoRI
SacI
HindIII
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EcoRI
MCS
pNHJ10H
(pUC19)
EcoRI
EcoRI
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(5.9kb)
(SmaI/EcoRV)
FspI
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SacI
EcoRI
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MCS
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XbaI, OII-in
FspI
ScaI
SacI
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SacI
EcoRI
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(XbaI/FipI)
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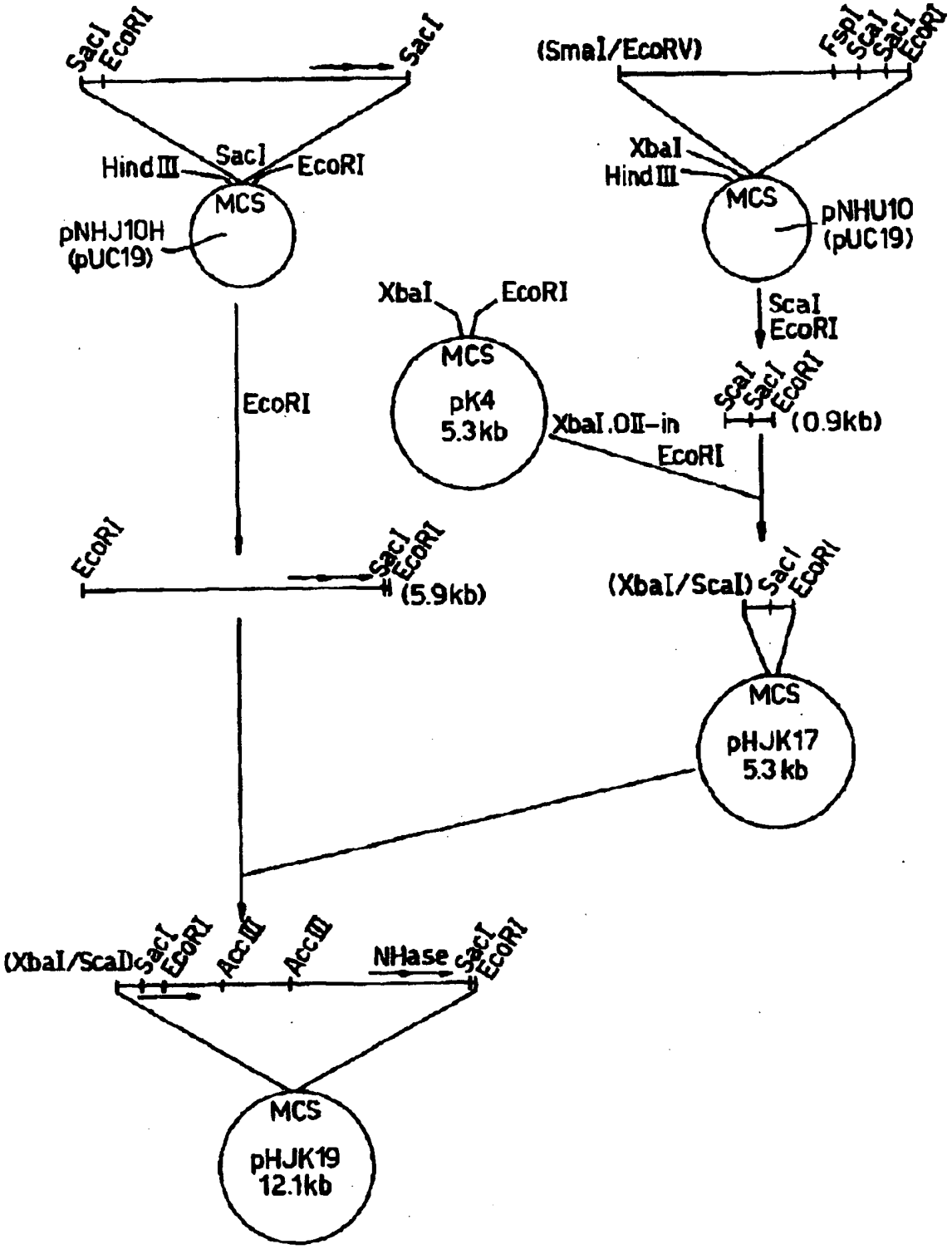
12.6kb

Fig.2.


SacI
EcoRI
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HindIII
SacI
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FspI
ScaI
SacI
EcoRI
XbaI
HindIII
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EcoRI
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(5.9kb)
(XbaI/ScaI)
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EcoRI
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EcoRI
AccIII
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NHase
SacI
EcoRI
MCS
pHJK19
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